Prevalence, molecular characterization and anti-microbial resistance pattern of *Staphylococcus* species isolates from buck semen

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Research Article

Keywords: Buck semen, Staphylococcus spp., CoNS, Methicillin resistance, Vancomycin resistance, reproductive health

Posted Date: September 28th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-919491/v1

Abstract

Staphylococcus aureus is one of the most prevalent pathogens, and a causative agent of a variety of infections in humans and animals. A total of 48 semen samples were collected from healthy bucks of different breeds to investigate the prevalence of S. aureus . Antimicrobial resistance and virulence of the Staphylococcus isolates were determined to assess the adverse effects of them on buck fertility. The bacterial isolates were tentatively confirmed as Staphylococcus spp. based on the Gram's staining, growth on Mannitol salt agar and catalase test. Overall, 75% (n = 36) of the samples were positive for Staphylococcus spp. from the total 48 buck semen ejaculates from different breeds. Out of 36 staphylococcal isolates, 23 (47.92%) were coagulase negative (CoNS) and 13 (27.08%) were coagulase positive Staphylococcus (CoPS) based on the slide coagulase test. In the current study, on the basis of molecular characterization, we identified S. aureus , S. chromogenes, S. haemolyticus, S. sciuri, S. simulans and S. epidermidis amongst the staphylococcal isolates in the buck semen. This study revealed a high prevalence of Staphylococcus species in semen of the healthy bucks. The isolates exhibited varying degrees of multidrug resistance genotypically as well as phenotypically. The presence of antibiotic resistance and virulence genes may pose a potential threat to reproductive health of animals, highlighting the need for vigilant monitoring of these isolates at the time of semen cryopreservation.

Highlights For Review

- The aim of the present study was to investigate the prevalence, and perform molecular characterization of *Staphylococcus* isolates from the semen samples of breeding bucks. This study involving the staphylococcus and its pathogenicity and Antimicrobial perspectives of buck semen are first of its kind.
- We evaluated the antimicrobial susceptibility, resistance genes and virulence genes of these isolates. Moreover, this type of work has not been carried out in goats.
- In the current study, the analysis showed, 75% (n = 36) of the samples were positive for *Staphylococcus* spp. from the total 48 buck semen ejaculates from different breeds. Out of 36 staphylococcal isolates, 23 (47.92%) were coagulase negative (CoNS) and 13 (27.08 %) were coagulase positive *Staphylococcus* (CoPS) based on the slide coagulase test. In the current study, on the basis of molecular characterization, we identified *S. aureus*, *S. chromogenes*
- Findings of this study throw light in the darkness of pathogenicity of *Staphylococcus spp.* present in buck-semen.

Introduction

Bacteria are present in every ejaculate of semen, and the aim to achieve sterility is paramount non-achievable task. Bacteria may gain access to the semen as a result of systemic infections due to bacteraemia, or due to local infections in parts of the genital tract, and inflammation genito-urinary tract. Another possible source can be due to the bacterial contamination during collection of ejaculate that gets

contaminated from the preputial saprophytic flora, external sources like fecal bacteria, environment and other substances of animal origin present in the diluents and buffers or equipment, human personnel etc. (Gangwar et al. 2020).

Staphylococcus aureus, a Gram-positive bacterium, is a causative agent of a variety of infections in humans and animals (McMillan et al. 2016). In animals, *S. aureus* is associated with mastitis, one of the most cost-intensive diseases in the dairy industry. Mastitis is an infectious disease responsible for significant financial losses to dairy and food farmers worldwide (Xing et al. 2016). Methicillin-resistant *S. aureus* (MRSA) was first reported in the United Kingdom, now it has become a public threat to human health, and various hospital-associated MRSA (HA-MRSA) clones have been disseminated globally (Monecke et al. 2011). Since the 1990s, community-associated MRSA (CA-MRSA) has emerged as a serious health problem in entire world (DeLeo et al. 2010). Livestock-associated MRSA (LA-MRSA) clones, such as LA-MRSA ST398 may be transmitted to humans, and have posed public health concerns (Fitzgerald 2012). Therefore, it is imperative to perform surveillance at the interface between human and animal hosts to explore human health risks (Cuny et al. 2015). Nowadays, the emergence of multidrug resistant (MDR) strains poses several challenges to the clinical facilities. In particular, the increasing prevalence of antimicrobial resistant *S. aureus* serves as a threat to the healthcare system (Hammad et al. 2012). On the other hand, most of *S. aureus* strains are able to produce a large number of virulence factors, including staphylococcal enterotoxins (SEs), exfoliative toxin (ET) and toxic shock syndrome toxin-1 (TSST-1). Moreover, the production of SEs is particularly significant, as the ingestion of the pre-formed toxins is a major cause of foodborne poisoning worldwide (Basanisi et al. 2017).

These pathogens, in one way or other, may infect inseminated females or contribute to a rapid deterioration in sperm quality (Al-Kass et al. 2019). Nevertheless, scientific evidence based on published data concerning the major risk pathogens or bacterial contamination of the germplasms used in goat AI operations in India, has been lacking until recently. The aim of the present study was to investigate the prevalence, and perform molecular characterization of *Staphylococcus* isolates from the semen samples of bucks. Furthermore, we evaluated the antimicrobial susceptibility, resistance genes and virulence genes of these isolates. Moreover, this type of work has not been carried out in goats. Findings of this study throw light in the darkness of pathogenicity of *Staphylococcus spp.* present in buck-semen.

Materials And Methods

2.1 Semen collection for bacteriological study

Total 48 semen ejaculates were collected from different goat breeds (Barbari, n= 12; Jamunapari, n= 12; Jakhrana, n= 12; Bundelkhandi, n=12) with good health condition, which were randomly selected during the breeding season, and maintained under semi-intensive system of rearing with same managemental conditions throughout this study. The whole experiment is carried out as per the guidelines of the Institute Animal Ethics Committee (IAEC).

2.2 Microbiological evaluation of semen

The fresh semen samples were inoculated into nutrient broth separately, and incubated at 37ºC overnight. The broth culture was inoculated on the nutrient agar and mannitol salt agar according to the method described by Mishra et al. (2014).

For identification of bacteria (*Staphylococcus aureus*), Gram's stain was performed according to the method described by Merchant and Packer (1967). For identifying coagulase positive staphylococci, coagulase test was performed using rabbit plasma as per the standard protocol. For coagulase test, a well isolated staphylococcal colony is to be emulsified in a drop of water on a clean and grease free glass slide with minimum of spreading. A flamed and cooled straight inoculating wire is dipped into the undiluted plasma at room temperature, and the adhering traces of plasma (not a loopful) is mixed into the staphylococcal suspension on the slide. In positive case, a coarse clumping of cocci is visible to the naked eye within 10 seconds.

2.4 Molecular characterization of *Staphylococcus* spp.

All the 36 isolates of *Staphylococcus* spp. were subjected to DNA extraction using Nucleo-pore Fungus Bacteria genomic DNA kit (Cat#NP-7006D) following the manufacturer's instructions. The DNA was checked for quality and quantity using Quantus™ fluorometer using the Quanti Fluor® ONEdsDNA system (E4871) following the manufacturer's instructions. Multiplex PCR was performed as described by Shome et al. (2011) with the oligonucleotide sequences and other details as described in the Table 1.

The primers are used in the specified concentration as mentioned above along with 2x Emerald GT amp master mix (TAKARA, Japan), along with 1µl template DNA (equivalent to approx. 2-3ng of bacterial genomic DNA) to make up the total volume of reaction to 25µl. The PCR was set-up with the following thermal conditions:

2.5 Anti-microbial resistance studies for *Staphylococcus* spp. isolates from buck semen

The confirmed coagulase negative *Staphylococci* (CoNS) and coagulase positive *Staphylococci* (CoPS) isolates were then subjected to β-Lactamase production and Methicillin resistant *Staphylococcus aureus* (MRSA) typing using both phenotypic and genotypic tests. All the antimicrobial tests were performed according to CLSI standards (CLSI, 2013) and their break-points.

2.5.1 β-Lactamase production in Staphylococcus spp by Penicillin zone edge test

This test was performed by using disk diffusion test by placing penicillin (10U) disk, and incubating at 35°C for overnight (16-18 hours) incubation. The tests are interpreted by observing the edges of inhibition zones which appear as sharp edge (cliff-like) as β-Lactamase positive and fuzzy edge (beach-like) as β-Lactamase negative.

2.5.2 Methicillin resistant Staphylococcus aureus (MRSA) typing using phenotypic tests

The phenotypic MRSA test was performed using the disk diffusion test employing cefoxitin disk (30 µg) for *S. aureus*, and CoNS. The inoculum was prepared, and spread on the Mueller Hinton agar (Oxoid, Thermofisher), and the antibiotic disk was placed over the inoculated plate after 15 min, and incubated at 35°C for 18 hours (*S. aureus*), and 24 hours (for CoNS). The cefoxitin disk (30 µg) with a minimum zone of inhibition (ZoI) of 22 mm and above is indicative of MRSA, and less than 22 mm is not indicative of MRSA. Similarly, for CoNS (except *S. pseudointermedius* and *S. schleiferi*) the ZoI should be ≥25mm for MRSA.

2.5.3 Methicillin resistant Staphylococcus aureus (MRSA) typing using Molecular methods

All the CoPS and CoNS isolates were subjected to genotypic MRSA detection using the genes viz., *mec*A and *mec*C (Table.2.). The primers used and their thermal cycling conditions are mentioned below.

The primers were used at a volume of 1µl (10pMol/µl) each of the forward and reverse primer along with 1µl template DNA (equivalent to approx. 2-3ng of bacterial genomic DNA), in 12.5µl of 2x Emerald GT amp master mix (TAKARA, Japan), and nuclease free water added in rest of volume to make up the total volume of reaction to 25µl. The PCR was set-up with the following thermal conditions illustrated below for both the MRSA genes.

2.5.4 Vancomycin resistant Staphylcoccus aureus (VRSA) detection by genotypic test.

All the isolates of *Staphylococcus* (n=36) were subjected to VRSA detection using two sets of genes viz., *VanA* and *Van*B. The details of the primers and their conditions are described in Table.2. The concentration and volume of the primers and reagents including the thermal conditions were similar to the previous section

Results

3.1: Isolation of *Staphylococcus* from Buck semen

The bacterial isolates were tentatively confirmed as Staphylococcus isolates based on the Gram's staining, growth on mannitol salt agar (Fig.1) and negative catalase test From the total 48 buck semen ejaculates from different breeds, 36 (75%) semen samples were positive for *Staphylococcus* spp. Out of 36 staphylococcal isolates, 23 (47.92%) were coagulase negative *Staphylococcus* (CoNS) and 13 (27.08 %) were coagulase positive *Staphylococcus* (CoPS) based on the slide coagulase test (Table.3).

3.2: Molecular characterization of *Staphylococcus* Spp.

After identification of bacteria as *Staphylococcus* spp., further classification of bacteria was done on the basis of molecular characterization (Fig.2). In the current study, we isolated *S. aureus*, *S. chromogenes, S. haemolyticus, S. sciuri, S. simulans* and *S. epidermidis* in the buck semen as given in the Table.5.

3.3. Phenotypic AMR typing

3.3.1 Detection of β-Lactamase producing *Staphylococcus* isolates by penicillin zone edge test

The Staphylococcus isolates were initially screened for the beta-lactamase activity using the penicillin zone edge test (Fig.3). Out of the 36 isolates tested, 7 (19.44%) produced cliff like zone edges suggestive of β-Lactamase positive isolates. Among the β-Lactamase producing *Staphylococcus* isolates, 2 were coagulase positive and 5 were coagulase negative.

3.3.2. Phenotypic detection of MRSA isolates.

MRSA typing of the isolates were conducted and the results are summarized in the Table.5 and depicted in Fig.3. Out of the 36 isolates, 23 (72.22%) were coagulase negative and 10 were coagulase positive (27.78%). From the total isolates tested (n=) 6 isolates (16.67%) were positive for MRSA by phenotypic test. Among the coagulase positive isolates, none of them tested positive for MRSA, while all the MRSA positive isolates were coagulase negative. Two (20.0%) of the coagulase negative *S. aureus* (n=10) and 4 (15.38%) of the CoNS (n=26) were tested positive for MRSA. Among the 5 coagulase negative- β-Lactamase producing *Staphylococcus* isolates screened (in previous section) 2 were also MRSA positive.

3.4: Genotypic Methicillin resistance and Vancomycin resistance typing

Among the methicillin resistant isolates, it may be either *S. aureus* or CoNS and hence those that were resistant, can be methicillin resistant *S. aureus* (MRSA) or methicillin resistant coagulase negative *Staphylococcus* (MRCoNS)

A total of 13 *S. aureus* and 23 CoNS were tested for methicillin resistance using the *Mec*A (Fig.4A) and *Mec*C gene (Fig.4B) conventional PCRs. Of the 13 isolates of *S. aureus*, 5 were positive for *Mec*A, and 4 were positive for *Mec*C. So, overall the MRSA isolates were 5 as per the *Mec*A gene which was on the higher side. As far as CoNS is concerned, 5 isolates and 2 isolates were detected for *Mec*A and *MecC*, respectively, and the overall MRCoNS isolates were 5 as per the *Me*cA which was on the higher side (Table.5).

Among the *S. aureus* isolates, 4 and 3 isolates were found positive for *Van*A and *Van*B genes (vancomycin resistant), respectively whereas, among the CoNS, 5 and 6 isolates were positive, respectively (Table.5 and Fig.4C).

Discussion

Infections of the male genitourinary tract may contribute to infertility by adversely affecting sperm function, causing inflammatory disorder, anatomical obstruction, alarmingly initiating leucocyte response with its concomitant oxidative stress (Gangwar et al. 2020). The effects of these conditions may be sperm damage, elevated leucocyte response, poor motility and immature sperms compromising the semen quality. Consequently it leads to compromise of sperm cell function and the whole

spermatogenesis (Gangwar et al. 2021). In the present study, we investigated the prevalence and performed molecular characterization of *Staphylococcus* spp. isolated from buck semen samples to facilitate better understanding of its prevalence in male genital tract and threat associated to it in buck fertility. The current study was conducted to assess the presence of *Staphylcoccus* species in the semen of bucks which were used for breeding. The semen analysis showed that the CoNS isolates were more in number (47.92%) compared to CoPS (27.08%). Among the *Staphylococcus* spp. isolates, both coagulase and non-coagulase producing bacteria are of equal importance with the growing pathogenic nature of the later (Zong et al. 2011).The CoNS are opportunistic in nature with all the pathogenic elements (Nasaj et al. 2020) and moreover the MRCoNS isolates are equally important in creating AMR hazard due to the harbouring of *SCCmec* elements in clinical isolates.

Staphylococcus aureus can colonize the reproductive tract affecting its functions (Kačániová et al. 2020) and the reproductive potential of spermatozoa. Most strains isolated from bull semen were *S. aureus* and *S. epidermidis*, and the metabolic products derived from these species were reported to have deleterious effects on the acrosomal integrity and motility (Meyer al. 1980). Earlier a study conducted on ejaculates of ram semen on bacteriological quality yielded *Staphylococcus aureus* and *Staphylococcus epidermidis* along with *E. coli*, *Enterobacter* spp., *Proteus mirabilis* in 97% of the samples when stored at 15°C (Yániz et al. 2010). In buffaloes, a similar study was conducted in the past revealed eleven different bacterial species in its semen including *S. aureus*, *S. intermedius*, *S. epidermidis* and coagulase negative *Staphylococcus* besides other gram-negative bacteria like *E. coli*, *Pseudomonas* etc. with the predominant bacteria being *S. aureus*. *S. aureus* affects the sperm function by hay wiring the metabolic processes (Boryczko, 1982), thereby reducing its viability. We found that the prevalence of *S. aureus* strains (Table. 1.) at 75 %, which was higher as compared to the previous reports (Liu et al. 2018). In the present study, *S. aureus*, *S. chromogenes*, *S. haemolyticus*, *S. sciuri*, *S. simulans*, *S. epidermidis* were isolated from the buck semen (Table. 3.). The influence of gram-positive uropathogenic bacteria on sperm morphology and function has been poorly investigated until now. A previous finding reported that aerobic cocci are present in about 50% of semen samples of male partners in infertile couples (Mehta et al. 2002). One of the most frequently isolated microorganisms from male animals with genital tract infections or semen contamination is *Escherichia coli* (Diemer et al. 2003) and *Staphylococcus* spp. (Enwurua et al. 2016). In the current study, besides *S. aureus*, we have obtained considerable number of isolates of *S. chromogenes*, *S. hemolyticus*, *S. simulans* and *S. sciuri* based on the multiplex PCR.

Staphylococcus aureus is amongst the most versatile and successful of the human pathogens and is capable of a wide spectrum of infections in the host. It colonizes the mucosal cavities, external orifices and skin surfaces and causes a variety of suppurative infections and toxic syndromes. Besides this, *S. aureus* is invariably involved in infertility among humans (Momoh et al. 2011). *S. aureus* has been observed as causative organism accounting for 68.2% of seminal fluid infections (Emokpae et al. 2009). Similar reports from Okon et al. (2005), where *S. aureus* was isolated from 62.5% of the seminal fluids. *S. aureus* has also been reported to be commonly isolated microorganism from cervical samples (Okonofua et al. 1995). Huwe et al. (1998) studied the influence of diferent uropathogenic microorganisms on human sperm motility parameters by means of CASA, and reported that *S. aureus* retards the sperm

motility. Similar study was also done previously by Liu et al. (2002) where significant decrease in sperm motility was found when spermatozoa were co-incubated with *S. aureus*. The reason behind the retarded motility is due to the inhibition of Mg++ ATPase activity of spermatozoa (Gupta and Prabha 2012). Some authors have suggested that direct interaction between bacteria and spermatozoa facilitates immobilization of spermatozoa (Nunez-Calonge et al. 1998), while others have reported evidence for soluble spermicidal factor produced and secreted by bacteria in the extracellular medium.

AMR is a very important aspect that requires immediate attention in any farm set up. Hence, in the current study, we screened all the isolates of *Staphylococcus* from buck semen for beta-lactamase production, methicillin resistance and vancomycin resistance. Among the coagulase positive isolates, none of them were MRSA positive, which was an interesting finding. On the other hand, among coagulase negative *S. aureus* isolates, a total of 6 isolates were found MRSA positive based on the cefoxitin disk diffusion phenotypic test. Similar patterns of MRSA in CoNS have been earlier reported in many instances (Li et al. 2021) and the horizontal transfer of AMR genes from less pathogenic *S. epidermidis* to *S. aureus* has been identified in certain studies during co-colonization (Li et al. 2021). But the genotypic test has revealed a totally different picture with good number of *S. aureus* (CoPS) carrying both *Mec*A and its homologue *Mec*C gene, besides the CoNS having equal number of MRSA isolates by the same test. Usually, the genes for resistance and other pathogenic traits of *Staphylococcus aureus* are present in the mobile genetic elements like pathogenicity islands, plasmids, transposons and staphylococcal chromosomal cassettes (SCCs) which may be exchanged in nature through various phenomena like conjugation, phage-aided transduction, transformation etc. (Wan et al. 2021). Similarly, methicillin resistance in *S. aureus* is coded in mobile genetic elements called staphylococcal cassette chromosome *mec* (SCC*mec*); where the methicillin resistant gene *Mec*A is present that code for the penicillin binding protein 2a. In the current study, as it can be seen that good number of isolates from buck semen were of coagulase negative origin, and a considerable proportion carried the *Mec*A as well as *Mec*C gene with some of them detected as phenotypic MRSAs. On these lines, the SCCs are transferred between staphylococci and there is a huge possibility that *Mec*A positive coagulase negative staphylococci may be a good source for exchange of these elements with pathogenic *S. aureus* as reported elsewhere (Hanssen and Ericson Sollid 2006). Although its effect on spermatozoa has been well established with spermicidal activity, reduction in sperm quality etc., but the post thaw effect of these MRSA especially during artificial insemination on breeding does is unknown. Moreover, the animal handlers who collect the semen, the artificial vagina and other equipments including the vaginal speculum need to be assessed for the presence of MRSA and MRCoNS, including the possibility of horizontal transmission of AMR and other mobile genetic elements from human-animal interaction.

*Mec*C is another important homologue of *Mec*A gene and found associated with several MRSA lineages including the most common lineage ‘CC130’ (Gómez et al. 2021). *Mec*C has been often associated with domestic animals (Gómez et al. 2014) including livestock like cattle and lactating cows (Schlotter et al. 2014) as well as wild animals (Loncaric et al. 2013), was considered to be zoonotic (Becker et al. 2014). Moreover, the presence of *Mec*C positive isolates in the current study especially in the reproductive tract of small ruminants could be treated as a concern that needs immediate control measures.

Notwithstanding, it is also important to study the lineages of these current isolates bearing the *Mec*C or *Mec*A to associate their origin and their transmission dynamics in the future.

Another important resistance is the vancomycin resistance, which was typed using the *Van*A and *Van*B genes. Vancomycin, a glycopeptide antibiotic is the drug of choice for treating patients complicated by MRSA infection, but the last few decades witnessed the development of intermediate (McGuinness et al. 2017) as well as completely resistant strains to vancomycin (Chang et al. 2003; Mukherjee et al. 2021). The current findings were similar to the MRSA genotyping with almost equal number positive for *Van*A and *Van*B in both *S. aureus* and CoNS isolates. The genotyping PCR gives first-hand information regarding the potential AMR characteristics of the isolates in a given entity. Also, these special genes might express given the apt environment leading to more damage by horizontal transfer of these genes to highly pathogenic isolates of *Staphylococcus*.

Generally, the prevalence of these bacteria is noteworthy as they may adhere to spermatozoa and decrease sperm motility (Prieto-Martínez et al. 2014). They can also damage acrosomes by producing toxin and cause DNA damage by producing reactive oxygen species (Morrell 2006). Eventually, this could result in decreased fertility rates and culling of breeding animals, and thus causing considerable financial losses to dairy industry (Moustacas et al. 2010). Hence, it is imperative to meticulously preclude the microbial contaminants from entering semen processing and storage facilities and AI operations (Eaglesome and Garcia 1997).

Conclusion

Artificial insemination is a very important tool in assisting reproductive technologies in livestock. The quality of the semen with respect to phenotypic and other functional analyses is well taken care of, while the microbial quality is a very important aspect that needs to be thoroughly addressed. Hence in this current study, we did the speciation and Anti-microbial resistance (AMR) analysis of staphylococcus isolates from buck semen. The AMR patterns could help decipher important information about the selection of antibiotics in semen dilutor and in turn could help assess the public health risk due to anti-microbial resistant isolates and their influence on personnel assisting the semen collection and processing for AI.

Declarations

Acknowledgements

The authors are thankful to the Director, ICAR-CIRG, Makhdoom, Mathura and INFAAR-FAO, New Delhi for facilitating the research work.

Authors contribution

Gururaj Kumaresan - Conceptualization of the work, Funding acquisition, writing the original draft**; Chetna Gangwar-** conceptualization, data curation, supervision and writing of manuscript its review and editing; **Anil Kumar** Mishra –investigations, methodology and validations; Ashok Kumar – supervision and project administration; **Suresh Dinkar Kharche -** supervision and project administration; **Narendra Pratap Singh** – Investigation and methodology; **Anjali Pachoori** – Formal analysis, visualization.

Conflict of Interest: Authors declare no conflict of interest.

References

1. Al-Kass Z., Eriksson E, Bagge E, Wallgren M, Morrell JM (2019) Bacteria detected in the genital tract, semen or pre-ejaculatory fluid of Swedish stallions from 2007 to 2017. Acta Vet Scand 61: 1-6. https://doi.org/10.1186/s13028-019-0459-z
2. Basanisi MG, La Bella G, Nobili G, Franconieri I, La Salandra G (2017) Genotyping of methicillin-resistant *Staphylococcus aureus* (MRSA) isolated from milk and dairy products in South Italy. Food Microbiol 62: 141-146. https://doi.org/10.1016/j.fm.2016.10.020
3. Becker K, Ballhausen B, Köck R, Kriegeskorte A (2014) Methicillin resistance in *Staphylococcus* isolates: the “mec alphabet” with specific consideration of mecC, a mec homolog associated with zoonotic *S. aureus* lineages. Int J Med Microbiol 304: 794-804. https://doi.org/10.1016/j.ijmm.2014.06.007
4. Bhatt P, Sahni AK, Praharaj AK, Grover N, Kumar M, Chaudhari C N, Khajuria A (2015) Detection of glycopeptide resistance genes in *Enterococci* by multiplex PCR. Med J Armed Forces India 71: 43-47 https://dx.doi.org/10.1016%2Fj.mjafi.2014.03.005
5. Boryczko Z (1982) Influence of *Pseudomonas aeruginosa, Staphylococcus aureus* and *Escherichia coli* types on metabolic processes and vitality of spermatozoa. Zuchthygiene 17: 13-18.
6. Chang S, Sievert DM, Hageman JC, Boulton ML, Tenover FC, Downes FP, Shah S, Rudrik JT, Pupp GR, Brown WJ, Cardo D, Fridkin SK (2003) Infection with vancomycin-resistant *Staphylococcus aureus* containing the vanA resistance gene. N Engl J Med 348: 1342-1347. https://dx.doi.org/10.1056/NEJMoa025025
7. CLSI (2013) Performance standards for antimicrobial disk and dilution susceptibility tests for bacteria isolated from animals VET01-S2. Second Information Supplement. Clinical and Laboratory Standards Institute, Wayne, PA.
8. Cuny C, Wieler LH, Witte W (2015) Livestock-associated MRSA: the impact on humans. Antibiotics 4: 521-543. https://doi.org/10.3390/antibiotics4040521
9. DeLeo F R, Otto M, Kreiswirth BN, Chambers HF (2010) Community-associated methicillin-resistant *Staphylococcus aureus*. The Lancet 375: 1557-1568. https://doi.org/10.1016/S0140-6736(09)61999-1
10. Diemer T, Huwe P, Ludwig M, Schroeder-Printzen I, Michelmann HW, Schiefer HG, Weidner W (2003) Influence of autogenous leucocytes and *Escherichia coli* on sperm motility parameters in vitro.

Andrologia 35: 100-105. doi:10.1046/j.1439-0272.2003.00523.x

11. Eaglesome MD, Garcia MM (1997) Disease risks to animal health from artificial insemination with bovine semen. Rev Sci Tech OIE 16: 215-225. https://doi.org/10.20506/rst.16.1.1017
12. Emokpae MA, Uadia PO, Sadiq NM (2009) Contribution of bacterial infection to male infertility in Nigerians. Online Journal of Health and Allied Sciences 8.
13. Enwurua CA, Iwalokuna B, Enwuru VN, Ezechi O, Oluwadun A (2016) The effect of presence of facultative bacteria species on semen and sperm quality of men seeking fertility care. Afr J Urol 22: 213-222. https://doi.org/10.1016/j.afju.2016.03.010
14. Fitzgerald JR (2012) Livestock-associated *Staphylococcus aureus*: origin, evolution and public health threat. Trends Microbiol 20: 192-198. https://doi.org/10.1016/j.tim.2012.01.006
15. Gangwar C, Kumaresan G, Mishra AK, Kumar A, Pachoori A, Saraswat S, Singh NP, Kharche SD (2020) Molecular detection of important abortion-causing microorganisms in preputial swab of breeding bucks using PCR-based assays. Reprod Domest Anim 55: 1520-1525. https://doi.org/10.1111/rda.13801
16. Gangwar C, Mishra AK, Gururaj K, Kumar A, Kharche SD, Saraswat S, Kumar R, Ramachandran N (2021) Semen quality and total microbial load: An association study in important Indian Goat breeds during different seasons. Andrologia 53: e13995. https://doi.org/10.1111/and.13995
17. Gómez P, González-Barrio D, Benito D, García JT, Viñuela J, Zarazaga M, Ruiz-Fons F, Torres C (2014) Detection of methicillin-resistant *Staphylococcus aureus* (MRSA) carrying the mecC gene in wild small mammals in Spain. J Antimicrob Chemother 69: 2061-2064. https://doi.org/10.1093/jac/dku100
18. Gómez P, Ruiz-Ripa L, Fernández-Fernández R, Gharsa H, Ben Slama K, Höfle U, Zarazaga M, Holmes MA, Torres C (2021) Genomic analysis of *Staphylococcus aureus* of the lineage CC130, including mecC-carrying MRSA and MSSA isolates recovered of animal, human, and environmental origins. Front Microbiol 12: 639. https://doi.org/10.3389/fmicb.2021.655994
19. Gupta S, Prabha V (2012) Human sperm interaction with *Staphylococcus aureus*: a molecular approach. J Pathog 2012. https://doi.org/10.1155/2012/816536
20. Hammad AM, Watanabe W, Fujii T, Shimamoto T (2012) Occurrence and characteristics of methicillin-resistant and-susceptible *Staphylococcus aureus* and methicillin-resistant coagulase-negative *Staphylococci* from Japanese retail ready-to-eat raw fish. Int J Food Microbiol 156: 286-289. https://doi.org/10.1016/j.ijfoodmicro.2012.03.022
21. Hanssen AM, Ericson Sollid JU (2006) SCC mec in *Staphylococci*: genes on the move. FEMS Microbiol Immunol 46: 8-20. https://doi.org/10.1111/j.1574-695X.2005.00009.x
22. Huwe P, Diemer T, Ludwig M, Liu J, Schiefer HG, Weidner W (1998) Influence of different uropathogenic microorganisms on human sperm motility parameters in an in vitro experiment. Andrologia 30: 55-59. https://doi.org/10.1111/j.1439-0272.1998.tb02827.x
23. Kačániová M, Terentjeva M, Štefániková J, Žiarovská J, Savitskaya T, Grinshpan D, Kowalczewski PŁ, Vukovic N, Tvrdá E (2020) Chemical composition and antimicrobial activity of selected essential oils

against *Staphylococcus* spp. isolated from human semen. Antibiotics 9: 765. https://doi.org/10.3390/antibiotics9110765

24. Li Y, Lin J, Li L, Cai W, Ye J, He S, Zhang W, Liu N, Gong Z, Ye X, Yao Z (2021) Methicillin-resistant coagulase-negative *Staphylococci* carriage is a protective factor of methicillin-resistant *Staphylococcus aureus* nasal colonization in HIV-infected patients: a cross-sectional study. Can J Infect Dis Med Microbiol 2021. https://doi.org/10.1155/2021/5717413
25. Liu B, Sun H, Pan Y, Zhai Y, Cai T, Yuan X, Gao Y, He D, Liu J, Yuan L, Hu G (2018) Prevalence, resistance pattern, and molecular characterization of *Staphylococcus aureus* isolates from healthy animals and sick populations in Henan Province, China. Gut Pathog 10: 1-13. https://doi.org/10.1186/s13099-018-0254-9
26. Liu JH, Li HY, Cao ZG, Duan YF, Li Y, Ye ZQ (2002) Influence of several uropathogenic microorganisms on human sperm motility parameters in vitro. Asian J Androl 4: 179-182.
27. Loncaric I, Kübber-Heiss A, Posautz A, Stalder G L, Hoffmann D, Rosengarten R, Walzer C (2013) Characterization of methicillin-resistant *Staphylococcus* spp. carrying the mecC gene, isolated from wildlife. J Antimicrob Chemother 68: 2222-2225. https://doi.org/10.1093/jac/dkt186
28. McGuinness WA, Malachowa N, DeLeo FR (2017) Focus: infectious diseases: vancomycin resistance in *Staphylococcus aureus*. Yale J Biol Med 90: 269.
29. McMillan K, Moore SC, McAuley CM, Fegan N, Fox E M (2016) Characterization of *Staphylococcus aureus* isolates from raw milk sources in Victoria, Australia. BMC Microbiol 16: 1-12. https://doi.org/10.1186/s12866-016-0789-1
30. Mehta RH, Sridhar H, Kumar BV, Kumar TA (2002) High incidence of oligozoospermia and teratozoospermia in human semen infected with the aerobic bacterium *Streptococcus faecalis*. Reprod Biomed Online 5: 17-21. https://doi.org/10.1016/S1472-6483(10)61591-X
31. Merchant IA, Packer RA (1967) Veterinary Bacteriology and Virology (7th ed.). The Iowa State University Press, Ames, Iowa, USA
32. Meyer M, Sonnenschein B, Bisping W, Krause D (1980) Pathogenic effect of metabolic products of *Staphylococcus aureus* strains on bull semen. Berl Munch Tierarztl 93: 66-74.
33. Mishra AK, Nitika S, Ashok K, Shivasharanappa N (2014) Prevalence of subclinical mastitis in different breeds of goats. Vet Pract 15: 140-141.
34. Momoh, ARM, Idonije BO, Nwoke EO, Osifo UC, Okhai O, Omoroguiwa A, Momoh AA (2011) Pathogenic bacteria-a probable cause of primary infertility among couples in Ekpoma. J Microbiol Biotechnol 1:66-71.
35. Monecke S, Coombs G, Shore AC, Coleman D C, Akpaka P, Borg M, Chow H, Ip M, Jatzwauk L, Jonas D, Kadlec K, Ehricht R (2011) A field guide to pandemic, epidemic and sporadic clones of methicillin-resistant *Staphylococcus aureus*. PloS One 6: e17936. https://doi.org/10.1371/journal.pone.0017936
36. Morrell J M (2006) Update on semen technologies for animal breeding. Reprod Domest Anim 41: 63-67. https://doi.org/10.1111/j.1439-0531.2006.00621.x

37. Moustacas VS, Xavier MN, Carvalho-Júnior CA, Costa EA, Henry M, Santos RL (2010) Effect of extender supplementation with various antimicrobial agents on viability of *Brucella ovis* and *Actinobacillus seminis* in cryopreserved ovine semen. Theriogenology 74: 1476-1481. https://doi.org/10.1016/j.theriogenology.2010.06.019
38. Mukherjee R, Priyadarshini A, Pandey RP, Raj VS (2021) Antimicrobial resistance in *Staphylococcus aureus*. Intech Open. https://doi.org/10.5772/intechopen.96888
39. Nasaj M, Saeidi Z, Asghari B, Roshanaei G, Arabestani MR (2020) Identification of hemolysin encoding genes and their association with antimicrobial resistance pattern among clinical isolates of coagulase-negative *Staphylococci*. BMC Res Notes 13: 1-6. https://doi.org/10.1186/s13104-020-4938-0
40. Nunez-Calonge R, Caballero P, Redondo C, Baquero F, Martinez-Ferrer M, Meseguer MA (1998). *Ureaplasma urealyticum* reduces motility and induces membrane alterations in human spermatozoa. Hum Reprod (Oxford, England) 13: 2756-2761. https://doi.org/10.1093/humrep/13.10.2756
41. Okon KO, Nwaogwu M, Zailani SO, Chama C (2005) Pattern of seminal fluid indices among infertile male partners attending the infertility clinic of university of Maiduguri teaching hospital, Maiduguri, Nigeria. Highl Med Res J 3: 18-23.
42. Okonofua FE, Ako-Nai KA, Dighitoghi MD (1995) Lower genital tract infections in infertile Nigerian women compared with controls. Sex Transm Infect 71: 163-168. http://dx.doi.org/10.1136/sti.71.3.163
43. Oliveira DC, Lencastre HD (2002) Multiplex PCR strategy for rapid identification of structural types and variants of the mec element in methicillin-resistant *Staphylococcus aureus*. Antimicrob Agents Ch 46: 2155-2161. https://doi.org/10.1128/AAC.46.7.2155-2161.2002
44. Prieto-Martínez N, Bussalleu E, Garcia-Bonavila E, Bonet S, Yeste M (2014). Effects of *Enterobacter cloacae* on boar sperm quality during liquid storage at 17 C. Anim Reprod Sci 148: 72-82. https://doi.org/10.1016/j.anireprosci.2014.05.008
45. Schlotter K, Huber-Schlenstedt R, Gangl A, Hotzel H, Monecke S, Müller E, Reißig A, Proft S, Ehricht R (2014) Multiple cases of methicillin-resistant CC130 *Staphylococcus aureus* harboring mecC in milk and swab samples from a Bavarian dairy herd. J Dairy Sci 97: 2782-2788. https://doi.org/10.3168/jds.2013-7378
46. Shome BR, Das Mitra S, Bhuvana M, Krithiga N, Velu D, Shome R, Isloor S, Barbuddhe SB, Rahman H (2011) Multiplex PCR assay for species identification of bovine mastitis pathogens. J Appl Microbiol 111:1349-1356. https://doi.org/10.1111/j.1365-2672.2011.05169.x
47. Stegger Á, Andersen PS, Kearns A, Pichon B, Holmes MA, Edwards G, Laurent F, Teale C, Skov R, Larsen AR (2012) Rapid detection, differentiation and typing of methicillin-resistant *Staphylococcus aureus* harbouring either mecA or the new mecA homologue mecALGA251. Clin Microbiol Infect 18: 395-400. https://doi.org/10.1111/j.1469-0691.2011.03715.x
48. Wan TW, Liu YJ, Wang YT, Lin YT, Hsu JC, Tsai JC, Chiu HC, Hsueh PR, Hung WC, Teng LJ (2021) Potentially conjugative plasmids harboring Tn6636, a multidrug-resistant and composite

mobile element, in *Staphylococcus aureus*. J Microbiol Immunol. https://doi.org/10.1016/j.jmii.2021.03.003

49. Xing X, Zhang Y, Wu Q, Wang X, Ge W, Wu C (2016) Prevalence and characterization of *Staphylococcus aureus* isolated from goat milk powder processing plants. Food Control 59: 644-650. https://doi.org/10.1016/j.foodcont.2015.06.042
50. Yániz JL, Marco-Aguado MA, Mateos JA, Santolaria P (2010) Bacterial contamination of ram semen, antibiotic sensitivities, and effects on sperm quality during storage at 15 C. Anim Reprod Sci 122: 142-149. https://doi.org/10.1016/j.anireprosci.2010.08.006

Tables

Table.1. List of oligonucleotides used along with details of amplicon size, concentration used in multiplex PCR for typing of *Staphylococcus* spp. (Shome et al. 2011)

Name of Gene	Oligonucleotide sequence 5'→3'	Size (bp)	Conc used (µM)	Species
23srRNA	F-AGCGAGTCTGAATAGGGCGTTT	894	0.25	*S. aureus*
	R-CCCATCACAGCTCAGCCTTAAC			
Nuc	F- GCGATTGATGGTGATACGGTT	278	0.8	*S.aureus*
	R- AGCCAAGCCTTGACGAACTAAAGC			
SodA	F-GCGTACCAGAAGATAAACAAACTC	222	0.2	*S. chromogenes*
	R-CATTATTTACAACGAGCCATGC			
SodA	F-CAAATTAAATTCTGCAGTTGAGG	214	0.2	*S. haemolyticus*
	R-AGAGCCCCATTGTTCTTTGA			
Gap	F-GATTCCGCGTAAACGGTAGAG	306	0.2	*S. sciuri*
	R-CATCATTTAATACTTTAGCCATTG			
Gap	F-AGCTTCGTTTACTTCTTCGATTGT	472	0.2	*S. simulans*
	R-AAAAGCACAAGCTCACATTGAC			
Rdr	F-AAGAGCGTGGAGAAAAGTATCAAG	130	0.2	*S. epidermidis*
	R-TCGATACCATCAAAAAGTTGG			

Table.2. Details of oligonucleotides used for MRSA and VRSA Genotyping PCR

S.No.	Name of the gene	Oligonucleotides (5'→3')	Product length	Reference
1.	*mec*A	*mec*AF: TCCAGATTACAACTTCACCAGG	162bp	Oliveira and Lencastre, (2002)
		*mec*AR: CCACTTCATATCTTGTAACG		
2.	*mec*C	*mec*CF: GAAAAAAAGGCTTAGAACGCCTC	138bp	Stegger et al. (2012)
		*mec*CR: GAAGATCTTTTCCGTTTTCAGC		
3.	*van*A	*vanAF:* GGGAAAACGACAATTGC	732bp	Bhatt et al. (2015)
		vanAR: GTACAATGCGGCCGTTA		
4.	*van*B	*van*BF: ACGGAATGGGAAGCCGA	647bp	
		*van*BR: TGCACCCGATTTCGTTC		

Table.3. Incidence of Staphylococcosis in buck semen

Breed	Total no of semen ejaculates tested	Semen ejaculates positive for Staphylococcus
Barbari	12	8 (66.67%)
Jamunapari	12	10 (83.33%)
Jakhrana	12	9 (75%)
Bundelkhandi	12	9 (75%)
Total	48	36 (75%)

Table.4. Molecular characterization of *Staphylococcus* spp. isolated from buck semen.

S.No.	Species typed by Multiplex PCR	Genes detected	No. of isolates screened
1.	*S. aureus*	*23SrRNA* (894bp), *Nuc* (278bp)	13
2.	*S. chromogenes*	*Sod*A (222bp)	11
3.	*S.hemolyticus*	*Sod*A (214bp)	2
4.	*S. sciuri*	*Gap* (306bp)	3
5.	*S.simulans*	*Gap* (472bp)	6
6.	*S.epidermidis*	*Rdr* (130bp)	1
		Total isolates	**36**

Table.5. Phenotypic and genotypic Anti-microbial resistance typing of CoPS and CoNS isolates

S.No.	Isolates	Phenotypic test				Genotypic tests			
		Coagulase positive		Coagulase negative		MRSA typing		VRSA typing	
		MRSA +ve	MRSA -ve	MRSA +ve	MRSA -ve	*Mec*A +ve	*Mec*C +ve	*Van*A +ve	*Van*B +ve
1.	*S. aureus* (n=13)	0	10	2	1	5	4	4	3
2.	CoNS (n=23)	-	-	4	19	5	2	5	6

Figures

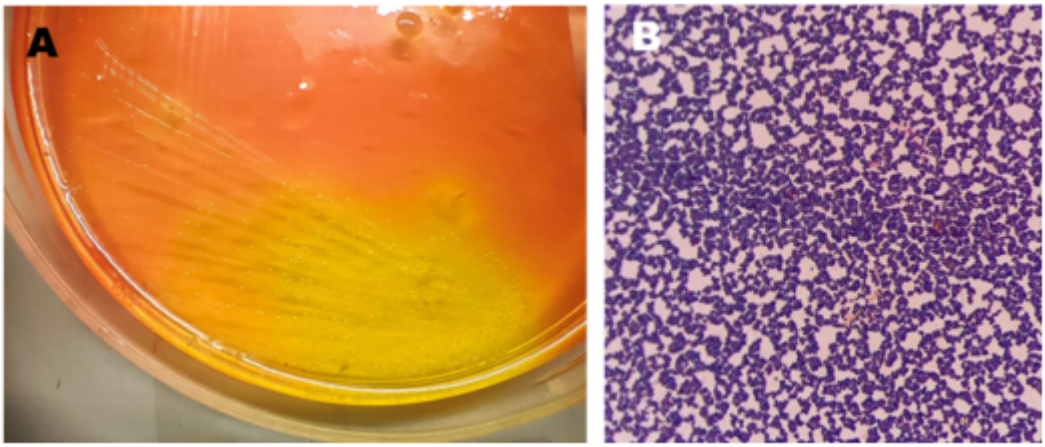

Figure 1

A- Growth of Staphylococcus aureus on mannitol salt agar with lemon yellow colonies surrounded by yellow zone suggestive of mannitol fermentation. B- Gram's stained smear of S. aureus appearing as Gram positive cocci arranged in bunches.

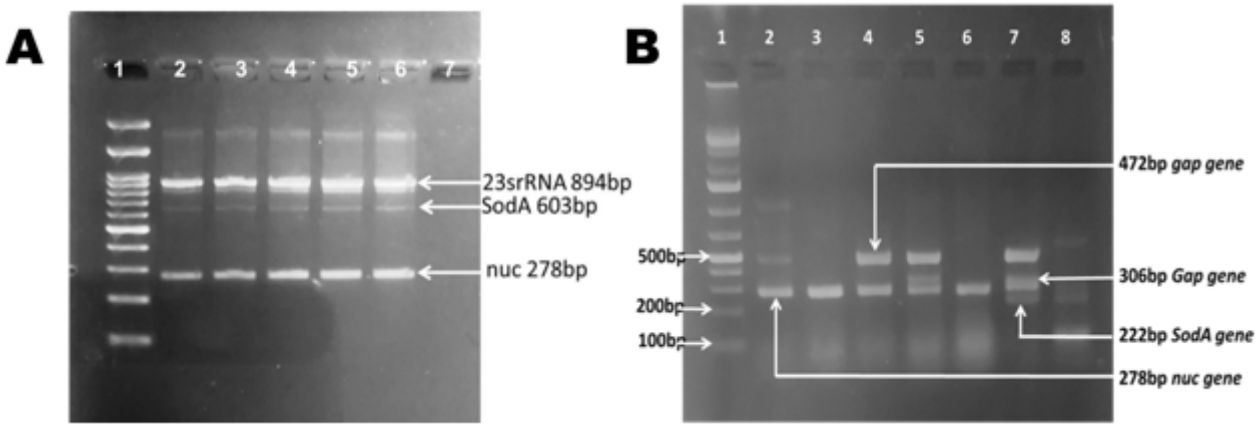

Figure 2

Staphylococcus speciation conventional multiplex PCR. A- Lanes, 1- 100bp DNA ladder, 2-6 nuc gene (278bp) and 23srRNA gene (894bp), 3-6 SodA gene (603bp), 7- No template control. B- Lanes, 1- 100bp DNA ladder, 2-7 nuc gene (278bp), 4-5 Gap gene (472bp), 5&7- Gap gene (306bp), 7- SodA (222bp) gene, 8-No template control

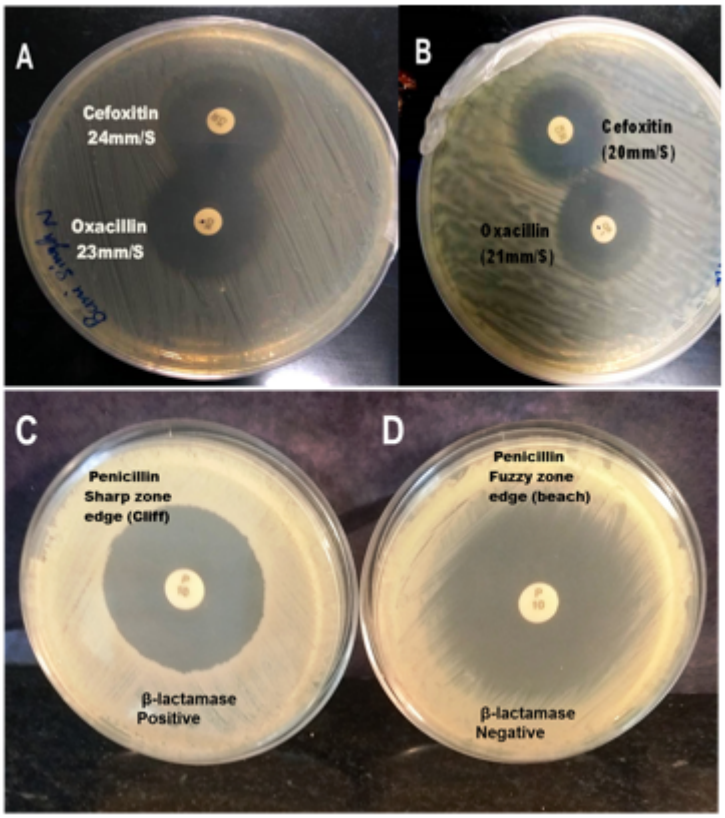

Figure 3

A- Coagulase positive Staphylococcus aureus isolate tested for MRSA production using cefoxitin and oxacillin disks. Tested MRSA negative based on cefoxitin inhibition zone (≥21mm); B- Coagulase negative staphylococcus (CoNS) isolate no. 298 tested for MRSA production using cefoxitin disk. The isolate tested MRCoNS positive based on Zone of inhibition (for CoNS, <24mm). C&D - β-Lactamase production in Staphylococcus spp. by Penicillin zone edge test. C- Left plate showing beta-lactamase production due to the 'cliff' like zone edge, D- right plate is beta-lactamase negative due to the 'beach' like zone edge.

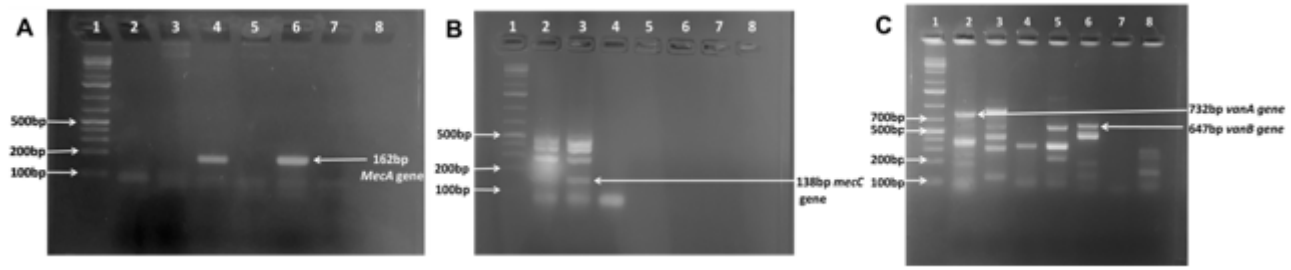

Figure 4

A- MRSA genotyping based on MecA gene conventional PCR. Lane 1- DNA Ladder, Lane 2-5 Unknown (Lane 4-positive- Mec A gene-162bp Isolate no.293 Goat semen), Lane 6- Positive control, Lane 7- No Template control. B- MRSA genotyping based on MecC gene conventional PCR. Lane 1- DNA Ladder, Lane

2-3 Unknown (positive for Mec C gene-138bp, Isolate no.293 Goat semen), Lane 4- No Template control. C- VRSA genotyping based on VanA/ VanB gene conventional PCR. Lane 1- 100bp DNA ladder, Lane 2&3- VanA gene (732bp), Lane 5-6- VanB gene (647bp), Lane 7&8– No template controls for VanA and VanB respectively.